Crosstalk between uterine serpin (SERPINA14) and pregnancy-associated glycoproteins at the fetal-maternal interface in pregnant dairy heifers experimentally infected with Neospora caninum

B. Serrano-Pérez[a,b], P.J. Hansen[c], R. Mur-Novales[a], I. García-Ispierto[a,b], N.M. de Sousa[d], J.F. Beckers[d], S. Almería[e], F. López-Gatius[b,*]

[a] Department of Animal Production, University of Lleida, 25198 Lleida, Spain

[b] Agrotecnio Centre, University of Lleida, Spain

[c] Department of Animal Sciences, University of Florida, USA

[d] Physiology of Reproduction, Faculty of Veterinary Medicine, University of Liège, Belgium

[e] Centre de Recerca en Sanitat Animal (CReSA), Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Universitat Autònoma de Barcelona (UAB), Barcelona, Spain

[f] Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona (UAB), Barcelona, Spain

[g] Transfer in Bovine Reproduction SLu, Barbastro, Spain

*Corresponding author: Tel: +34 073 702563; fax: +34 973 238264; email address: flopez@prodan.udl.cat (F. López-Gatius)

Abstract

Infection with *Neospora caninum* is the leading cause of abortion in cattle. In cows naturally infected with *N. caninum*, plasma concentrations of pregnancy-associated glycoproteins (PAG) 1 and 2 indicate fetal-placental well-being, whereas an excess of progesterone in the second trimester of gestation has been related to high abortion rate. The immunosuppressive action of progesterone on the uterus during gestation has been attributed in part to the uterine serpins (SERPINA14). This study examines expression patterns of the genes *SERPINA14*, *PAG1*, and *PAG2* at the fetal-maternal interface in dairy heifers experimentally infected with *N. caninum* during the second trimester of pregnancy, when most abortions takes place in natural conditions. Irrespective of infection, expression of *SERPINA14* was higher, and expression of *PAG1* and *PAG2* lower, for intercaruncular endometrium than for caruncles or cotyledons. Cotyledonary tissues showed the highest expression of both PAG genes but lowest expression of *SERPINA14*. The expression of *SERPINA14* was significantly higher in intercaruncular endometrium of control dams than for infected animals, pointing to potential disruption of modulation of maternal immune function during infection. Dramatically reduced *SERPINA14* was particularly apparent in infected dams with aborted fetuses. There was also a negative association between *N. caninum* antibody titers with *SERPINA14* and PAG expression in infected animals, further suggesting that *N. caninum* infection downregulates the uterine immunosuppressive function of SERPINA14.

Keywords: Bovine neosporosis; Placenta well-being; Immune modulation of gestation

1. Introduction

Neospora caninum is considered a main cause of abortion in cattle worldwide [1–4]. *Neospora*-seropositive cows carry a 12 to 19 times greater risk of abortion than seronegative cows; the incidence of abortion ranges from 30% to 44% in seropositive animals [5,6]. Further, the risk of repeat abortion persists in seropositive cows [7]. The major route of *N. caninum* infection in dairy herds is transplacental, meaning that the parasite passes from dams to their fetuses during pregnancy [8,9]. Maternal immunity, host susceptibility, parasite strain diversity, and the stage of fetal development at which infection is acquired have all been related to transplacental infection and abortion [10–12]. Parasites may provoke lesions in the placenta that are severe enough to cause fetal death and pregnancy termination [3,4]. Among several cell-mediated immunity mechanisms, those induced by T helper 1 cells (Th1) have been described as the most important for reducing parasite multiplication in the host [2,12]. However, Th1 activity, although effective in nonpregnant animals, could play a role in the pathogenesis of fetal rejection during gestation [13].

Pregnancy-associated glycoproteins (PAGs) are a multigene family related to aspartic proteinases that are expressed in the placenta of artiodactyls. Ruminant PAGs are classified into two main groups: one of ancient origin (the PAG-II subgroup, including PAG2, PAG8, PAG10, PAG11, PAG12, PAG12, and PAG22), largely occurring at the placental fetal-maternal interface, and one produced by a more recent series of gene duplications (the PAG-I subgroup, including PAG1, PAG3, PAG15, PAG17, and PAG21), expressed primarily in trophoblast binucleate cells [14,15]. Plasma PAG-I concentrations are unaffected by chronic *N. caninum* infection in dams although PAG-I

and PAG-II concentrations in aborting animals are useful indicators of fetal-placental distress [16–18].

In high-producing dairy cows, *Neospora* infection affects endocrine patterns during gestation such that *Neospora* seropositivity has been associated with higher plasma progesterone (P4) concentrations [19]. Progesterone, a key pregnancy hormone, participates in the natural immunomodulation of gestation, reducing the Th1 response to induce maternal immunologic tolerance to the fetus [20,21]. However, excess P4 in the second trimester of gestation leads to a higher abortion rate in cows chronically infected with *N. caninum* [22]. Thus, a threshold Th1 immune response such as gamma interferon production seems necessary to confer protection against abortion [23]. The maternal immune system can therefore tolerate the presence of paternal alloantigens without affecting antiinfection mechanisms during gestation. The uterine immunosuppressive actions of P4 have been attributed in part to uterine serpins [24]. These basic glycoproteins are members of the serpin superfamily of serine peptidase inhibitors. One such member, SERPINA14 [25], is expressed in response to P4 in the endometrium. According to its uterine expression and loss of proteinase inhibitory activity, a new function in establishing and maintaining pregnancy in ruminants has been suggested [26]. SERPINA14 inhibits lymphocyte function in vitro [27] and selectively interacts with other uterine proteins, such as PAGs [28], uteroferrin [29], IgM and IgA [30], and activin [31]. SERPINA14 is not only secreted by the endometrium of the pregnant ruminant it is also present in fetal fluids (allantoic and amniotic fluids) [32] and ovarian luteal and follicular structures [33].

The present study is one of a series of investigations performed in pregnant dairy heifers experimentally infected with *N. caninum*. The objective was to examine expression of SERPINA14, PAG1, and PAG2 genes at the fetalmaternal interface in the second trimester of gestation of infected animals. This stage of pregnancy was selected as the time when most abortions occur in field conditions. Also assessed were possible interrelations between expression patterns of SERPINA14 and PAG with plasma *N. caninum* antibodies and plasma concentrations of PAG-I and PAG-II in the infected dams.

2. Material and methods

2.1. Animals and infection

A full description of the parasite inocula used and the characteristics of the experimentally infected heifers is provided by Almeria et al. (unpublished data, 2015). Briefly, ten 14 to 16-month-old Holstein-Friesian heifers that were seronegative against *N. caninum* (CIVTEST, Spain) were synchronized and artificially inseminated. Seronegativity against the parasite was assessed before artificial insemination and on Days 60 and 90 of gestation. Heifers were previously vaccinated (6–8 months of age) against bovine viral diarrhea virus and infectious bovine rhinotracheitis virus. Pregnancy was assessed by ultrasound at 30, 45, 90, and 110 days after insemination. On Day 110 of gestation, six of the heifers were intravenously inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7, kindly donated by Dr. L.M. Ortega-Mora (SALUVET, Universidad Complutense, Madrid, Spain). These six animals were euthanized on Day 152 of gestation. The four remaining heifers remained

as uninoculated controls and euthanized at the same time as inoculated dams. After Day 110, heifers were visually inspected daily for possible abortion until their sacrifice.

2.2. Sample collection

Blood samples for antibody and placental protein determinations were collected from each heifer by tail vein puncture into heparinized vacuum tubes (BD Vacutainer, Becton-Dickinson and Company, Plymouth, UK) on Day 152 of gestation. Plasma obtained by centrifugation within 30 minutes of sampling was stored at -20 °C until analysis.

On Day 152 of gestation (6 weeks after infection), all animals were sedated with xylazine hydrochloride (Rompun; Bayer) and euthanized by an intravenous overdose of embutramide and mebezonio iodide (T61; Intervet). Immediately after death, heifers were necropsied. Amniotic and allantoic fluids were collected before the placenta was opened, and fetuses separated from the placenta. Fetal blood samples were obtained by cardiac puncture. Samples of nine selected placentomes (three cranial, three medial, and three caudal) were removed from each dam. Both the maternal side of the placenta (caruncle) and its corresponding fetal side (cotyledon) were carefully separated manually from each placentome. Intercaruncular tissue was also collected. Tissues collected from fetuses were CNS (brain and spinal cord), heart, lung, liver, skeletal muscle, spleen, and thymus.

2.3. Ethics

All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and University of Lleida (license number CEEA.06-01/12). Animals were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee of the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

2.4. Sample analyses

2.4.1. Plasma antibodies against *Neospora caninum*

Plasma samples collected from the dams and fetuses were tested for anti-*Neospora caninum* antibodies using a commercial ELISA kit based on the whole tachyzoite lysate of *Neospora* NC-1, according to the manufacturers' instructions (CIVTEST® anti-*Neospora*; Hipra, Girona, Spain). The cut-off used for a positive test result was an value of 6.0 absorbance units, as previously established [6]. Fetal fluids were analyzed using the same technique on undiluted samples.

2.4.2. Polymerase chain reaction (PCR)-based diagnosis of *Neospora caninum*

Portions of placenta and fetal tissues were aseptically obtained and stored in liquid nitrogen at -196ºC until DNA extraction. At least 0.5–1 g of each tissue were homogenized with a pestle and mortar in liquid nitrogen and DNA extracted as described by Almeria et al. [34].

171 2.4.3. PAG-1 and PAG-2 determination

172

173 Pregnancy-associated glycoprotein 1 concentrations were determined in plasma using a
174 double antibody radioimmunoassay procedure (RIA-706) [35]. As the primary antibody,
175 rabbit polyclonal antiserum AS#706 raised against caprine $PAG_{55\ kDa+62\ kDa}$ (accession
176 numbers P80935 and P80933) was used [36]. The minimum detection limit for the RIA
177 procedure was 1.2 ng/mL. Intra- and inter-assay coefficients of variation were 5.3% and
178 6%, respectively.

179

180 The bovine PAG-II RIA procedure used has been recently described [16,37]. Briefly,
181 PAG-2 was purified according to the method detailed by Beckers et al. [38]. The
182 primary antibody was rabbit polyclonal antiserum against boPAG-2 (AS#438) raised
183 according to the method of Vaitukaitis [36]. Owing to the instability of the boPAG-2
184 molecule, boPAG-1 (67 kDa) was used as a standard (dilutions ranging from 100 to 0.2
185 ng/mL) and for iodination with the 125-I isotope [39]. The initial dilution for primary
186 AS#438 was 1:2500. The minimal detection limit calculated for RIA-438 was 2.3
187 ng/mL. Intra- and inter-assay coefficients were 4.6% and 4.8%, respectively.

188

189 2.4.4. RNA extraction and gene expression

190 Intercaruncular and placental tissue samples were kept frozen in liquid nitrogen,
191 homogenized in a mortar in the presence of additional liquid nitrogen and maintained in
192 trizol (Invitrogen Corp., Carlsbad, CA, USA) at -80ºC. For caruncle or cotyledon tissue
193 gene expression analysis, a mixed sample of RNA from the three different sections
194 (cranial, medial and caudal) of each tissue was used as template.

195

Total RNA was extracted according to the method of Chomczynski and Sacchi [40]. Samples were treated with DNAse in the presence of RNAse inhibitors to eliminate contaminating genomic DNA. Concentrations of RNA were determined spectrophotometrically, and RNA integrity was checked by denaturing agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from 4 µg of total RNA in the presence of random primers using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's recommendations.

Messenger RNA expressionwas determined by real time RT-PCR for three target genes: serpin peptidase inhibitor, clade A member 14 *(SERPINA14)*, *PAG1*, and *PAG2*. The genes b-actin *(ACTB)* and ribosomal protein L19 *(RPL19*) were used as housekeeping genes. Sequences of primers for *PAG1* and *PAG2* were those described by Touzard et al. [41]. Primers used for *ACTB* have been published elsewhere [42]. Primers for RPL19 (forward primer: 5'-GATCCGGAAGCTGATCAAAG-3, reverse primer: 5-ATTCGAGCATTGGCAGTACC-3') and SERPINA14 (Access number NM_174797; forward primer: 5'- TTTGGAGGCCCTACATCAAG-3, reverse primer: 5-GACCTCCTTTGCCTTCATTG-3') were designed with Primer3Plus tool (www.bioinformatics.nl/primer3plus) and synthesized by Isogen-Life Sciences (Isogen-Life Science B.V., De Meern, The Netherlands). To avoid genomic contamination, all the primers were designed to span an intron. For each gene, we generated a standard curve by amplifying serial dilutions of a control cDNA to check for linearity between initial template concentration and cycle threshold (Ct) values. Amplification were conducted using the SYBR green method with the ABI PRISM^{TM} 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) under the conditions specified by

the manufacturer: an initial activation and denaturation step of 10 min at 95 °C followed by 40 cycles consisting of 10 seconds at 95 °C and 1 minute at 60 °C. Polymerase chain reactions were run using 3 μL of 30- fold diluted cDNA as template in a total volume of 8 μL containing 1× Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Inc., MD, USA), and 200 nM of forward and reverse primers. Each measurement was carried out in triplicate and the average used to calculate the relative amount of gene.The $2^{-\Delta\Delta Ct}$ was used for data normalization and analysis employing as calibrator a pool of RNA from the tissues used in this study [42].

2.5. Statistical analyses

One-way analysis of variance was used to compare relative *SERPINA14*, *PAG1*, and *PAG2* expression for maternal (caruncle and intercaruncle) and fetal (cotyledon) sides of the placenta and among groups (uninfected controls, infected dams with aborted fetuses, and infected dams with live fetuses). When significant differences were detected, the Bonferroni test was used to examine all possible pairwise comparisons. Spearman's rho (sr) test was used to identify possible relationships between gene expression levels of *SERPINA14*, *PAG1*, and *PAG2* and *Neospora* seropositivity or plasma PAG-I and PAG-II concentrations before euthanasia in controls and infected nonaborting animals. All tests were performed using the computer package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Significance was set at $P \leq 0{\cdot}05$.

3. Results

3.1. Fetal viability

Fetal death was recorded in 3 of 6 infected dams: two dams aborted 14 and 21 days after infection, and a third dam showed a mummified fetus upon euthanasia. Pathologic findings in these three fetuses are described in Almeria et al. (unpublished data, 2015). For this study, placentomes were not available or were in a too poor condition for analysis in the aborted fetuses.

3.2. Plasma antibody response

Anti-*N. caninum* antibodies were detected in plasma from all infected dams, whereas control heifers (n = 4) remained seronegative. *N. caninum* antibodies were also detected in serum (two fetuses), allantoic fluid (three fetuses), and amniotic fluid (two fetuses) collected from the infected dams.

3.3. Detection of *N. caninum* DNA

N. caninum DNA was observed in at least one tissue type and in the placentas (caruncles and/or cotyledons) from all recovered infected fetuses (S. Almería et al, unpublished data, 2015). All DNA samples from control, uninfected fetuses were negative.

3.4. Gene expression for *SERPINA14*, *PAG1*, and *PAG2*

Significantly higher ($P = 0.006$) expression of *SERPINA14* and significantly lower ($P < 0.001$) expression of *PAG1* and *PAG2* were observed in intercaruncular tissues when compared with cotyledon and caruncle samples (Fig.1A–C). Highest expression levels of *PAG1* and *PAG2* were observed in the cotyledons compared with caruncular or intercaruncular tissues ($P < 0.01$) (Fig. 1B, C). In maternal tissues (caruncle vs.

intercaruncle), significant differences were observed for the expression of *PAG1* (P = 0.013; Fig. 1B) but not *PAG2* (Fig. 1C).

The expression of *SERPINA14* in intercaruncular tissues was higher for control (uninfected) dams as compared with infected animals (P = 0.001; Fig. 2A). Infected dams carrying a live fetus showed higher *PAG1* expression in intercaruncular tissues compared with that for control or aborting infected dams (P = 0.017). The expression of *PAG2* was higher for infected dams carrying a live fetus compared with that for aborting infected dams (P = 0.027) but not for control dams (Fig. 2B). No differences were detected in *SERPINA14*, *PAG1*, or *PAG2* expression between the caruncles and cotyledons of nonaborting infected dams versus control dams.

Neospora seropositivity was negatively correlated with *SERPINA14* expression in intercaruncular (r: -0.786, P = 0.036) and cotyledon samples (r: -0.786, P = 0.036) and positively correlated with *PAG1* expression in intercaruncular tissues (r: 0.821, P = 0.023).

3.5. Plasma PAG concentrations

Significantly higher plasma PAG-I and PAG-II concentrations were observed in both control and nonaborting infected dams versus aborting infected animals on euthanasia (P = 0.001 and P = 0.004, respectively; Fig. 3).

In controls and nonaborting infected dams, SERPINA14 expression in the cotyledons was negatively associated with plasma PAG-II concentrations (sr: -0.929, P = 0.003).

294 The expression of *PAG1* in caruncles was positively associated with plasma PAG-1
295 concentrations (sr: 0.857, P = 0.014), whereas *PAG2* expression in cotyledons was
296 positively correlated with plasma PAG-I and PAG-II concentrations (sr: 0.857, P =
297 0.014 and sr: 0.750; P = 0.052, respectively).
298
299 4. Discussion
300
301 In this report, we characterize immune-endocrine responses in bovine neosporosis by
302 examining the expression of *SERPINA14*, *PAG1* and *PAG2* expression at the fetal-
303 maternal interface following experimental *N. caninum* infection in pregnant dairy
304 heifers. Our main findings were that: (1) irrespective of infection, intercaruncular,
305 caruncular, and cotyledonary tissue samples showed different degrees of expression for
306 *SERPINA14*, *PAG1*, and *PAG2*; (2) *N. caninum* infection affected the expression of all
307 three genes in intercaruncular endometrium; (3) S*ERPINA14* expression in
308 intercaruncular endometrium was negatively correlated with *PAG1* and *PAG2*
309 expression levels and negatively correlated with antibody production against *N.*
310 *caninum*; and (4) *PAG1* and *PAG2* expression were positively correlated with plasma
311 concentrations of both PAGs and antibody production against *N. caninum*.
312
313 Greater *SERPINA14* expression was observed in intercaruncular tissue than placentome
314 tissue, in agreement with the findings of other authors [44]. On the contrary, the lowest
315 expression levels of PAGs were detected in the intercaruncular area, whereas
316 placentomes showed the highest *PAG* expression levels, especially in cotyledonary
317 tissues. The segregation of modern PAGs in cotyledons and of ancient PAGs in the
318 intercotyledonary chorion suggests their distinct biological functions within placental

319 tissues [41]. The differences observed between caruncular and cotyledonary tissues also
320 indicate good tissue separation in our study.
321
322 The experimental infection with *N. caninum* on Day 110 of gestation clearly modified
323 the expression of *SERPINA14*, *PAG1*, and *PAG2* in intercaruncular tissues.
324 Unfortunately, we could not obtain intercotyledonary tissue samples in aborted and/or
325 mummified fetuses. Uterine serpins can inhibit a wide variety of lymphocyte functions
326 such as mitogen-induced proliferation and natural killer cytotoxicity [45]. The
327 significantly higher expression of *SERPINA14* observed here in the intercaruncular
328 tissues of control dams point to a role of this molecule in maternal immune modulation
329 of gestation. However, the dramatically reduced *SERPINA14* expression detected in
330 infected animals, especially in aborting dams, and the negative correlation observed
331 between *SERPINA14* and *PAG* expression along with antibody production suggest that
332 *N. caninum* infection is able to downregulate *SERPINA14* expression. One possible
333 consequence would be to prevent possible antiproliferative actions of the serpin on *N.*
334 *caninum* growth.
335
336 It may also be, however, that reduced *SERPINA14* expression during infection
337 represents a maternal immunologic adjustment to enhance maternal immune responses
338 against *Neospora* by reducing local immunosuppression. It may also be that disruption
339 of pregnancy leads to a reduction in the expression of many pregnancy-associated
340 uterine proteins. This could be tested by examining the expression of some other P4-
341 induced uterine proteins. The interaction of this molecule with cellular and humoral
342 immune responses against *N. caninum* infection remains to be clarified.

As we anticipated, *PAG* expression in placentome tissues showed positive correlation with plasma PAG concentrations in control and nonaborting infected dams. Despite the low *PAG* expression observed in intercaruncular tissues compared with the levels detected in the placentomes, *N. caninum* infection significantly upregulated PAG expression in this endometrial area in dams with live fetuses compared to uninfected controls. As noted for plasma PAG levels, *PAG* expression in the intercaruncular tissues of aborting infected dams was practically undetectable, probably because of uterine tissue damage caused by parasite multiplication. We hypothesize that a strong Th1 immune response after multiplication of *N. caninum* and reduction in immunosuppression caused by reduced *SERPINA14* expression provokes severe lesions in the placenta and endometrium causing the death of the fetus.

As argued by Wallace et al. [46], the accumulation of binucleated trophoblast cells (cells related to the secretion of the modern [type I] PAGs) in the maternal uterine stroma would position them to potentially influence lymphocyte or PMN leukocyte migration and/or activation. Thus, the positive correlation observed between *PAG1* expression and *N. caninum* antibody production could reflect parasite replication in the endometrium of the nonaborting infected heifers.

In summary, our findings indicate differential expression of *SERPINA14*, *PAG1*, and *PAG2* in intercaruncular and placental tissues. The dramatic decrease in *SERPINA14* expression detected in infected dams, particularly in those with aborted fetuses, and the negative correlation observed between the expression of *SERPINA14*, *PAG1*, and *PAG2* expression with *N. caninum* antibody production in infected animals seem to indicate

367 that *N. caninum* infection can directly or indirectly regulate the expression of
368 *SERPINA14* so that uterine immunosuppression is reduced.
369
370 Acknowledgments
371 This study was supported by a grant from the Spanish MINECO (AGL2012-39830-
372 C02-01/02) and FEDER. Ramón Mur-Novales was awarded an FPI grant by the
373 Spanish Ministry of Science and Innovation, MICINN, BES-2013-063215. The authors
374 thank Ana Burton for editorial assistance, the farmers who provided the experimental
375 animals and the staff of CReSA for their help with managing the animals, and Dr. L.M.
376 Ortega-Mora (SALUVET, Universidad Complutense, Madrid, Spain), for the *Neospora*
377 isolate.
378
379 References
380
381 [1] Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Vet
382 Parasitol 1996;67:1-59.
383 [2] Dubey JP, Schares G, Ortega-Mora LM. Epidemiology and control of neosporosis
384 and *Neospora caninum*. Clin Microbiol Rev 2007;20(2):323-67.
385 [3] Dubey JP, Schares G. Neosporosis in animals-the last five years. Vet Parasitol
386 2011;180:90-108.
387 [4] Almería S, López-Gatius F. Bovine neosporosis: clinical and practical aspects. Res
388 Vet Sci 2013;95:303-9.
389 [5] López-Gatius F, López-Béjar M, Murugavel K, Pabón M, Ferrer D, Almería S.
390 *Neospora*-associated abortion episode over a 1-year period in a dairy herd in north-east
391 Spain. J Vet Med B Infect Dis Vet Public Health 2004;51:348-52.

392 [6] López-Gatius F, Pabón M, Almería S. *Neospora caninum* infection does not affect
393 early pregnancy in dairy cattle. Theriogenology 2004;62:606-13
394 [7] Mazuz ML, Fish L, Reznikov D, Wolkomirsky R, Leibovitz B, Savitzky I, Golenser
395 J, Shkap V. Neosporosis in naturally infected pregnant dairy cattle. Vet Parasitol
396 2014;205:85-91.
397 [8] Schares G, Peters M, Wurm R, Bärwald A, Conraths FJ. The efficiency of vertical
398 transmission of *Neospora caninum* in dairy cattle analysed by serological techniques.
399 Vet Parasitol 1998;80(2):87-9.
400 [9] Davison HC, Otter A, Trees AJ. Estimation of vertical and horizontal transmission
401 parameters of *Neospora caninum* infections in dairy cattle. Int J Parasitol
402 1999;29(10):1683-9.
403 [10] Williams DJ, Guy CS, McGarry JW, Guy F, Tasker L, Smith RF, MacEachern K,
404 Cripps PJ, Kelly DF, Trees AJ. *Neospora caninum*-associated abortion in cattle: the
405 time of experimentally-induced parasitaemia during gestation determines foetal
406 survival. Parasitology 2000;121:347-58.
407 [11] Gibney EH, Kipar A, Rosbottom A, Guy CS, Smith RF, Hetzel U, Trees AJ,
408 Williams DJ. The extent of parasite-associated necrosis in the placenta and foetal tissues
409 of cattle following *Neospora caninum* infection in early and late gestation correlates
410 with foetal death. Int J Parasitol 2008;38:579-88
411 [12] Almería S, López-Gatius F. Markers related to the diagnosis and to the risk of
412 abortion in bovine neosporosis. Res Vet Sci 2015;100:169-75.
413 [13] Hansen PJ. Maternal immunological adjustments to pregnancy and parturition in
414 ruminants and possible implications for postpartum uterine health: is there a prepartum-
415 postpartum nexus? J Anim Sci 2013;91:1639–49.

416 [14] Wooding FB, Roberts RM, Green JA. Light and electron microscope
417 immunocytochemical studies of the distribution of pregnancy associated glycoproteins
418 (PAGs) throughout pregnancy in the cow: possible functional implications. Placenta
419 2005;26:807-27.
420 [15] Garbayo JM, Serrano B, López-Gatius F. Identification of novel pregnancy-
421 associated glycoproteins (PAG) expressed by the periimplantation conceptus of
422 domestic ruminants. Anim Reprod Sci 2008;103:120-34.
423 [16] López-Gatius F, Garbayo JM, Santolaria P, Yániz JL, Almería S, Ayad A, de
424 Sousa NM, Beckers JF. Plasma pregnancy- associated glycoprotein-1 (PAG-1)
425 concentrations during gestation in *Neospora*- infected dairy cows. Theriogenology
426 2007;67:502-8.
427 [17] García-Ispierto I, Almería S, Serrano B, de Sousa NM, Beckers JF, López-Gatius
428 F. Plasma concentrations of pregnancy-associated glycoproteins measured using anti-
429 bovine PAG-2 antibodies on day 120 of gestation predict abortion in dairy cows
430 naturally infected with *Neospora caninum*. Reprod Domest Anim 2013;48:613-8.
431 [18] García-Ispierto I, Serrano-Pérez B, Almería S, Martínez-Bello D, Tchimbou AF, de
432 Sousa NM, Beckers JF, López-Gatius F. Effects of crossbreeding on endocrine patterns
433 determined in pregnant beef/dairy cows naturally infected with *Neospora caninum*.
434 Theriogenology. 2015;83:491-6.
435 [19] García-Ispierto I, Nogareda C, Yániz JL, Almería S, Martínez-Bello D, de Sousa
436 NM, Beckers JF, López-Gatius F. *Neospora caninum* and *Coxiella burnetii*
437 seropositivity are related to endocrine pattern changes during gestation in lactating dairy
438 cows. Theriogenology 2010;74:212-20.
439 [20] Druckmann R, Druckmann MA. Progesterone and the immunology of pregnancy. J
440 Steroid Biochem Mol Biol 2005;97:389-96.

441 [21] Szekeres-Bartho J, Barakonyi A, Par G, Polgar B, Palkovics T, Szereday L.
442 Progesterone as an immunomodulatory molecule. Int Immunopharmacol 2001;1:1037-
443 48.
444 [22] Bech-Sàbat G, López-Gatius F, Santolaria P, García-Ispierto I, Pabón M, Nogareda
445 C, Yániz JL, Almería S. Progesterone supplementation during mid-gestation increases
446 the risk of abortion in *Neospora*-infected dairy cows with high antibody titres. Vet
447 Parasitol 2007;145:164-7.
448 [23] López-Gatius F, Almería S, Donofrio G, Nogareda C, García-Ispierto I, Bech-
449 Sàbat G, Santolaria P, Yániz JL, Pabón M, de Sousa NM, Beckers JF. Protection against
450 abortion linked to gamma interferon production in pregnant dairy cows naturally
451 infected with Neospora caninum. Theriogenology 2007;68:1067-73.
452 [24] Hansen PJ, Ing NH, Moffatt RJ, Baumbach GA, Saunders PT, Bazer FW, Roberts
453 RM. Biochemical characterization and biosynthesis of the uterine milk proteins of the
454 pregnant sheep uterus. Biol Reprod 1987;36:405-18.
455 [25] Ing NH, Roberts RM. The major progesterone-modulated proteins secreted into the
456 sheep uterus are members of the serpin superfamily of serine protease inhibitors. J Biol
457 Chem 1989;264:3372-9.
458 [26] Padua MB, Kowalski AA, Cañas MY, Hansen PJ. The molecular phylogeny of
459 uterine serpins and its relationship to evolution of placentation. FASEB J 2010;24:526-
460 33.
461 [27] Padua MB, Hansen PJ. Regulation of DNA synthesis and the cell cycle in human
462 prostate cancer cells and lymphocytes by ovine uterine serpin. BMC Cell Biol 2008;9:5.
463 [28] Mathialagan N, Hansen TR. Pepsin-inhibitory activity of the uterine serpins. Proc
464 Natl Acad Sci U S A 1996;93:13653-8.

465 [29] Baumbach GA, Ketcham CM, Richardson DE, Bazer FW, Roberts RM. Isolation
466 and characterization of a high molecular weight stable pink form of uteroferrin from
467 uterine secretions and allantoic fluid of pigs. J Biol Chem 1986;261:12869-78.
468 [30] Hansen PJ, Newton GR. Binding of immunoglobulins to the major progesterone-
469 induced proteins secreted by the sheep uterus. Arch Biochem Biophys 1988;260:208-
470 17.
471 [31] McFarlane JR, Foulds LM, O'Connor AE, Phillips DJ, Jenkin G, Hearn MT, de
472 Kretser DM. Uterine milk protein, a novel activin-binding protein, is present in ovine
473 allantoic fluid. Endocrinology. 1999;140:4745-52.
474 [32] Newton GR, Hansen PJ, Bazer FW, Leslie MV, Stephenson DC, Low BG.
475 Presence of the major progesterone-induced proteins of the sheep endometrium in fetal
476 fluids. Biol Reprod 1989;40:417-424.
477 [33] Ulbrich SE, Frohlich T, Schulke K, Englberger E, Waldschmitt N, Arnold GJ,
478 Reichenbach HD, Reichenbach M, Wolf E, Meyer HH, Bauersachs S. Evidence for
479 estrogen-dependent uterine serpin (SERPINA14) expression during estrus in the bovine
480 endometrial glandular epithelium and lumen. Biol Reprod 2009;81:795-805.
481 [34] Almería S, Ferrer D, Pabón M, Castellà J, Manas S. Red foxes (Vulpes vulpes) are
482 natural intermediate host of *Neospora caninum*. Vet Parasitol 2002;107;287-94.
483 [35] López-Gatius F, Garbayo JM, Santolaria P, Yániz J, Ayad A, de Sousa NM,
484 Beckers JF. Milk production correlates negatively with plasma levels of pregnancy-
485 associated glycoprotein (PAG) during the early fetal period in high producing dairy
486 cows with live fetuses. Domest Anim Endocrinol 2007;32:29-42.
487 [36] Vaitukaitis J, Robbins JB, Nieschlag E, Ross GT. A method for producing specific
488 antisera with small doses of immunogen. J Clin Endocrinol Metab 1971;33:988–91.

489 [37] Serrano-Pérez B, Garcia-Ispierto I, de Sousa N, Beckers J, Almería S, López-
490 Gatius F. Gamma interferon production and plasma concentrations of pregnancy-
491 associated glycoproteins 1 and 2 in gestating dairy cows naturally infected with
492 *Neospora caninum*. Reprod Domest Anim 2014;49:275-80.
493 [38] Beckers JF, Dewulf M, Verstegen J, Wouters-Ballman P, Ectorsm F. Isolation of a
494 bovine chorionic gonadotrophin (bCG). Theriogenology 1988;29:218. Abstract.
495 [39] Thorell JI, Johansson BG. Enzymatic iodination of polypeptides with 125I to high
496 specific activity. Biochim Biophys Acta 1971;251:363-9.
497 [40] Chomczynski P, Sacchi N. Single-step method of RNA isolationby acid
498 guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 1987;162:156-9.
499 [41] Touzard E, Reinaud P, Dubois O, Guyader-Joly C, Humblot P, Ponsart C,
500 Charpigny G. Specific expression patterns and cell distribution of ancient and modern
501 PAG in bovine placenta during pregnancy. Reproduction 2013;146:347-62.
502 [42] Ribeiro ES, Bruno RG, Farias AM, Hernández-Rivera JA, Gomes GC, Surjus R,
503 Becker LF, Birt A, Ott TL, Branen JR, Sasser RG, Keisler DH, Thatcher WW, Bilby
504 TR, Santos Low doses of bovine somatotropin enhance conceptus development and
505 fertility in lactating dairy cows. JE Biol Reprod 2014;90:10.
506 [43] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-
507 time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-8.
508 [44] Stephenson DC, Leslie MV, Low BG, Newton GR, Hansen PJ, Bazer FW.
509 Secretion of the major progesterone-induced proteins of the sheep uterus by caruncular
510 and intercaruncular endometrium of the pregnant ewe from days 20–140 of gestation.
511 Domest Anim Endocrinol 1989;6:349–62.
512 [45] Tekin S, Padua MB, Brad AM, Hansen PJ. Antiproliferative actions of ovine
513 uterine serpin. Am J Reprod Immunol. 2005; 53:136-43.

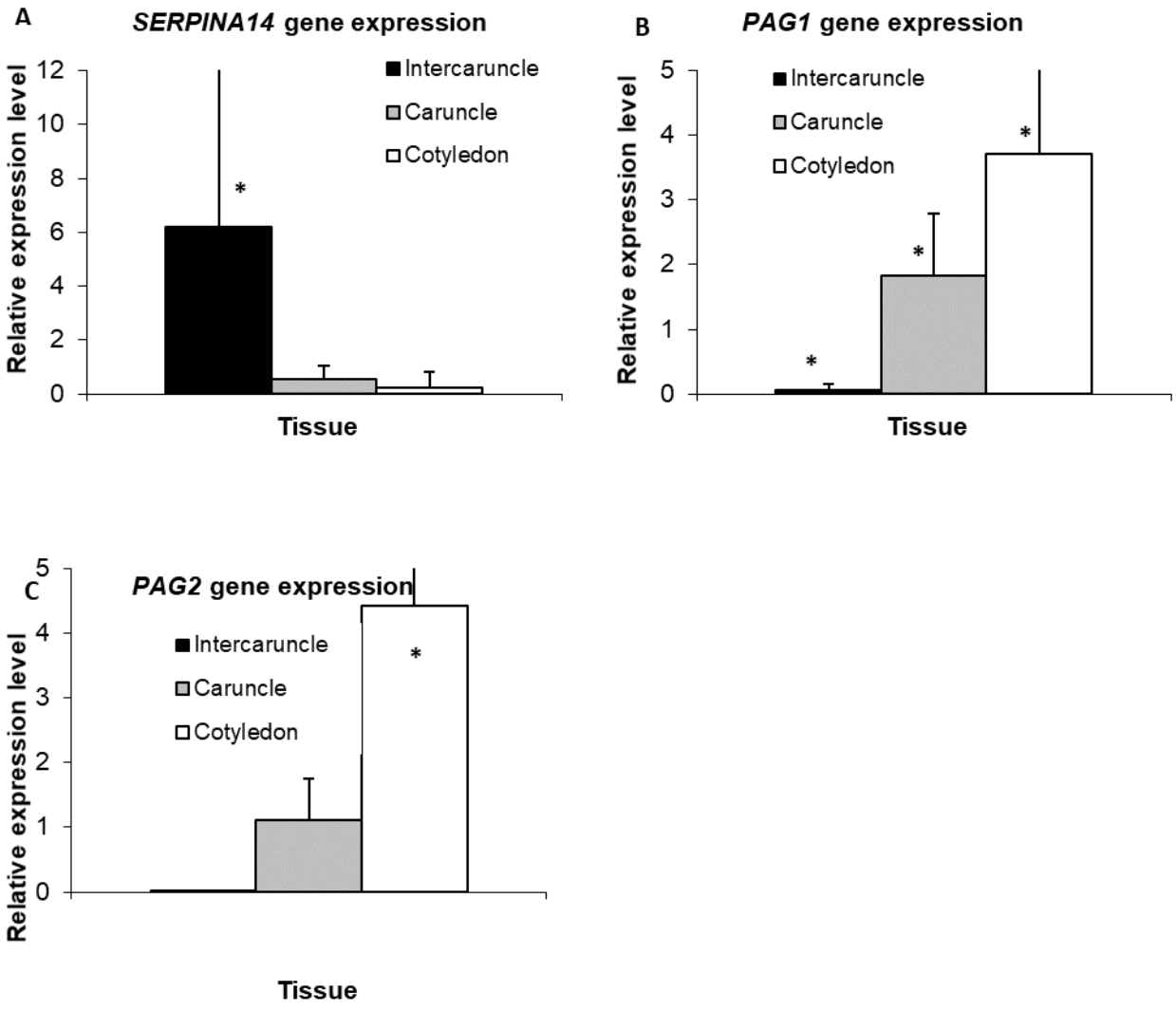
514 [46] Wallace RM, Pohler KG, Smith MF, Green JA. Placental PAGs: gene origins,
515 expression patterns, and use as markers of pregnancy. Reproduction. 2015;149:R115-
516 26.
517

518 Figures

519

520 Figure 1. Relative expression of *SERPINA14* (A), *PAG1* (B) and *PAG2* (C) in the
521 cotyledons (n=7), caruncle (n=8) and intercaruncular tissues (n=10) of control and
522 experimenta dairy heifers infected with *Neospora caninum* on Day 152 of gestation.
523 Bars represent mean values ± standard error of the mean. *Values for each tissue were
524 different according to the Bonferroni test (P<0.05). PAG, pregnancy-associated
525 glycoproteins.

526

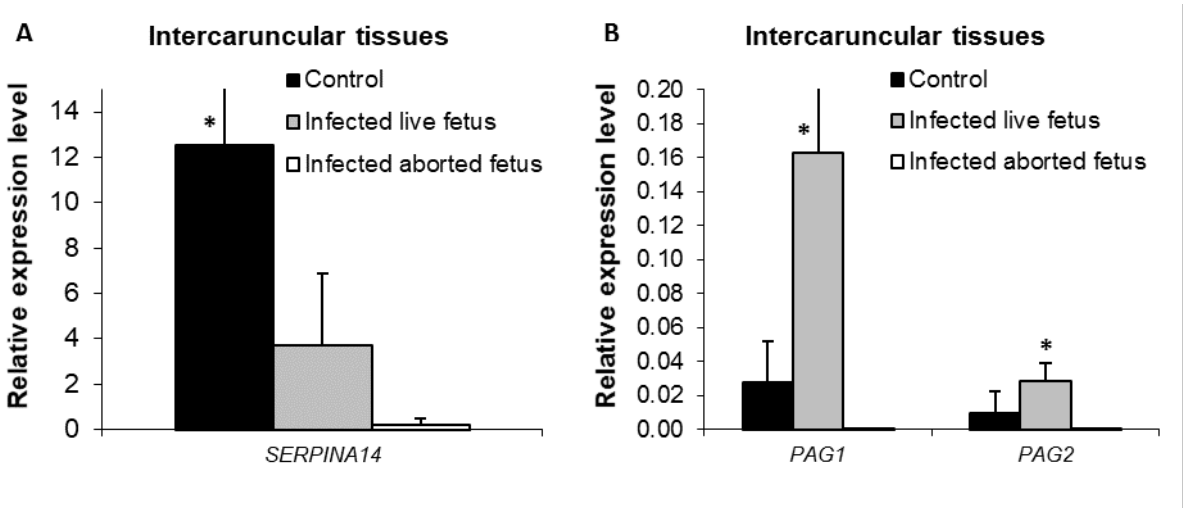


527

528

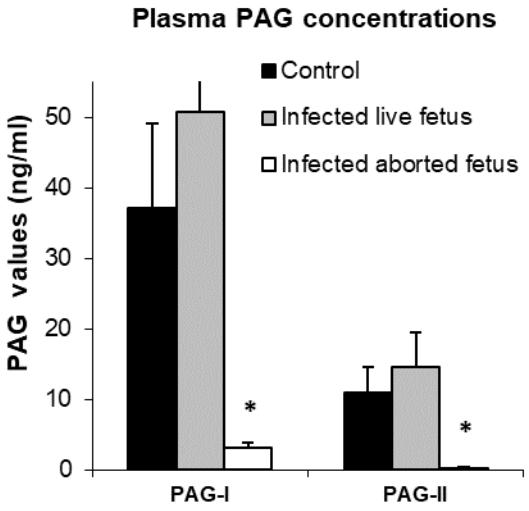
529 Figure 2. Relative gene expression levels of *SERPINA14* (A), and *PAG1* and *PAG2* (B)
530 in intercaruncular tissues samples from: heifers experimentally infected with *Neospora*
531 *caninum* on Day 152 of gestation; infected heifers carrying aborted and/or nonviable
532 fetuses on Day 42 after infection (n = 3); live fetuses (n = 3); and control uninfected
533 animals (n = 4). Bars represent the mean ± standard error of the mean. *Values for each
534 group differing according to the Bonferroni test (P < 0.05).

535



536
537

538 Figure 3. Plasma PAG-I and PAG-II concentrations recorded in: heifers infected with
539 Neospora caninum on Day 42 after infection, infected heifers carrying aborted and/or
540 nonviable fetuses (n = 3); infected heifers carrying live fetuses (n = 3), and control
541 uninfected animals (n = 4). Bars represent the mean ± standard error of the mean.
542 *Values of each variable differing according to the Bonferroni test (P < 0.05).



543

544

545

546